

Attenuation of Delayed Neuronal Death After Mild Focal Ischemia in Mice by Inhibition of the Caspase Family

Matthias Endres, Shobu Namura, Masao Shimizu-Sasamata, Christian Waeber, Lin Zhang,
 *Teresa Gómez-Isla, *Bradley T. Hyman, and Michael A. Moskowitz

*Stroke and Neurovascular Regulation and *Alzheimer Research Group, Neurosurgery and Neurology Department, Massachusetts General Hospital, Harvard Medical School, Charlestown, Massachusetts, U.S.A.*

Summary: Inhibitors of apoptosis and of excitotoxic cell death reduce brain damage after transient and permanent middle cerebral artery occlusion. We compared the neuroprotective effects of two caspase family inhibitors with the *N*-methyl-D-aspartate receptor antagonist (+)-MK-801 hydrogen maleate (MK-801) in a newly characterized cycloheximide-sensitive murine model of transient middle cerebral artery occlusion (30 minutes) in which apoptotic cell death is prominent. Ischemic infarction, undetected by 2,3,5-triphenyltetrazolium chloride staining at 24-hour reperfusion, featured prominently in the striatum at 72 hours and 7 days on hematoxylin-eosin-stained sections. Markers of apoptosis, such as oligonucleosomal DNA damage (laddering) and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL)-positive cells first appeared at 24 hours and increased significantly at 72 hours and 7 days after reperfusion. The TUNEL-labeled cells were mostly neurons and stained negative for glial (GFAP, glial fibrillary acid protein) and leukocyte specific markers (CD-45). The caspase inhibitors, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD.FMK; 120 ng intracerebroventricularly) or *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone (z-DEVD.FMK; 480 ng intracerebroventricularly) decreased infarct size and neurologic deficits when administered 6 hours

after reperfusion. The extent of protection was greater than in models of more prolonged ischemia or after permanent occlusion, and the therapeutic window was extended from 0 to 1 hours after 2-hour middle cerebral artery occlusion to at least 6 hours after brief ischemia. Also, z-VAD.FMK and z-DEVD.FMK treatment decreased oligonucleosomal DNA damage (DNA laddering) as assessed by quantitative autoradiography after gel electrophoresis. By contrast, MK-801 protected brain tissue only when given before ischemia (3 mg/kg intraperitoneally), but not at 3 or 6 hours after reperfusion. Despite a decrease in infarct size after MK-801 pretreatment, the amount of DNA laddering did not decrease 72 hours after reperfusion, thereby suggesting a mechanism distinct from inhibition of apoptosis. Hence, 30 minutes of reversible ischemia augments apoptotic cell death, which can be attenuated by delayed z-VAD.FMK and z-DEVD.FMK administration with preservation of neurologic function. By contrast, the therapeutic window for MK-801 does not extend beyond the time of occlusion, probably because its primary mechanism of action does not block the development of apoptotic cell death. **Key Words:** interleukin-1 β converting enzyme family caspases—Apoptosis—Cerebral ischemia—Delayed neuronal death—Mice.

Received June 2, 1997; final revision received September 11, 1997; accepted September 13, 1997.

Supported by Massachusetts General Hospital Interdepartmental Stroke Project Grants (NS10828) and an unrestricted award in Neuroscience from Bristol Myers Squibb (M.A. Moskowitz). Dr. M. Endres was supported by the Deutsche Forschungsgemeinschaft (En343/1-1), Dr. S. Namura by the Uehara Memorial Foundation, and Drs. T. Gómez-Isla and B. Hyman were supported by NIH (AG05134 and AG11337) and a generous gift from the Walters Family Foundation.

Address correspondence and reprint requests to Dr. Michael A. Moskowitz, Neurosurgery and Neurology Department, Massachusetts General Hospital, Harvard Medical School, 149 13th Street, Room 6403, Charlestown, MA 02129, U.S.A.

Abbreviations used: DMSO, dimethylsulfoxide; GFAP, glial fibrillary acid protein; H&E, hematoxylin-eosin; ICE, interleukin-1 β converting enzyme; MCAO, middle cerebral artery occlusion; MK-801, (+)-MK-801 hydrogen maleate; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; rCBF, regional cerebral blood flow; TTC, 2,3,5-triphenyltetrazolium chloride; TTC, triphenyltetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; z-DEVD.FMK, *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone; z-VAD.FMK, *N*-benzyloxycarbonyl-ylVal-Ala-Asp-fluoromethyl ketone.

Several laboratories provide evidence for cell death by an apoptotic mechanism in animal models of cerebral ischemia (Li et al., 1995a,b,c; MacManus et al., 1995a,b; Charriaut-Marlangue et al., 1996). Apoptosis is a cell suicide program under active cell control. The interleukin-1 β converting enzyme (ICE) family caspases, implicated in apoptotic cell death (Yuan and Horvitz, 1990; Ellis et al., 1991), are the human homologues of the nematode *Caenorhabditis elegans* CED-3. Eleven members of this family have been identified, which share a common QACXG consensus sequence (Alnemri et al., 1996). Caspase 1 (ICE) cleaves a pro-interleukin-1 β 31-kilodalton (kd) protein to generate mature 17.5-kd interleukin-1 β , which is involved in inflammatory reactions (Dinarello, 1994), apoptosis (Friedlander et al., 1996), as well as focal and global cerebral ischemia (Saito et al., 1996; Liu et al., 1993). Caspase 3 (CPP32), another important family member implicated in apoptotic cell

death, cleaves poly(ADP-ribose)polymerase and DNA-dependent protein kinase delta, among other substrates (Tewari et al., 1995; Nicholson et al., 1995).

Reports from our laboratory show that peptide inhibitors of the caspase family (*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone [z-VAD.FMK] and *N*-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone [z-DEVD.FMK]) are neuroprotective after focal reversible cerebral ischemia in mice and rats (Hara et al., 1997a). Both drugs reduced tissue injury and associated neurologic deficits after 2-hour middle cerebral artery occlusion (MCAO) when assessed at 24 and 72 hours (Hara et al., 1997a). Interleukin-1 β formation, a product of ICE cleavage, was significantly blocked by z-VAD.FMK, a rather unselective caspase inhibitor, but not by the putative caspase 3 inhibitor z-DEVD.FMK. Smaller infarcts also were observed in transgenic mice expressing a dominant negative mutation of ICE (Friedlander et al., 1997; Hara et al., 1997b). Cleavage of pro-interleukin-1 β is important to the development of ischemic damage (Relton and Rothwell, 1992; Yamasaki et al., 1995) as is a cascade that may involve activation of caspase 3. We speculated that an important mechanism by which z-VAD and z-DEVD decrease infarct size relates to blockade of ischemia-induced apoptosis and in this report, we tested the effects of peptide methylketones in a murine model of brief (30 minutes) transient focal cerebral ischemia.

Mild injury augments apoptotic cell death in in vitro and in vivo models of cell injury, including brain ischemia. In the rat, infarct development is delayed until 3 days after 30 minutes of distal middle cerebral artery occlusion whereas tissue damage develops much earlier (within 24 hours) after 90 minutes or 2 hours of reversible MCAO. In this rat model, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells and ladder DNA are present in the peri-infarct zone, suggesting a role for apoptotic cell death (Du et al., 1996).

In the current study, we developed a model of mild ischemia in mice showing delayed infarct development and delayed appearance of several apoptosis markers (TUNEL staining, DNA laddering). We then evaluated the efficacy and the treatment window of two caspase inhibitors. We determined whether inhibition of caspase family members by the use of peptide methylketones decreases DNA laddering in ischemic tissue as measured by densitometry on agarose gels. Delayed therapeutic intervention—possibly directed against apoptotic death—may become an important therapeutic strategy to reduce mild ischemic insults (Du et al., 1996).

METHODS

Drugs

Both z-VAD.FMK and z-DEVD.FMK were obtained from Enzyme Systems Products (Dublin, CA, U.S.A.). The com-

pounds were dissolved in 0.3% dimethylsulfoxide (DMSO; MC/B, Norwood, OH, U.S.A., prepared with 0.1 M phosphate-buffered saline [PBS] pH 7.4). Cycloheximide (Chex) was purchased from Sigma (St. Louis, MO, U.S.A.) and dissolved in PBS. We obtained (+)-MK-801 hydrogen maleate (MK-801) from Research Biochemicals International (Natick, MA, U.S.A.) and dissolved it in PBS.

Physiology

Regional cerebral blood flow (rCBF) was measured by Laser-Doppler flowmetry (PF2B, Perimed, Stockholm, Sweden) along with arterial blood pressure and heart rate as described (Hara et al., 1996, 1997a,b). Arterial blood samples (50 μ L) were analyzed for pH, oxygen (Pao₂), and carbon dioxide (Paco₂) using a blood gas/pH analyzer (Corning 178, Ciba-Corning Diagnostics, Medford, MA, U.S.A.). Core temperature was maintained at 36.5° \pm 1°C with a temperature control unit (FHC, Brunswick, ME, U.S.A.) and a heating lamp (Skytron, Daiichi Shomei, Tokyo, Japan) until 1 hour after reperfusion and during the treatment and monitoring period. With the exception of MK-801 experiments (see treatment protocol), animals were kept at room temperature during the reperfusion period. For physiologic monitoring, animals (selected randomly) were monitored for rCBF and physiologic parameters before and during ischemia, and until 30 minutes after reperfusion for different reperfusion protocols (24 and 72 hours; *n* = 4 per group). In an earlier study, we determined that intracerebroventricular pretreatment with peptide methylketones had no obvious effects on rCBF, mean arterial blood pressure, heart rate, core temperature, and arterial blood gases (Hara et al., 1997a).

Ischemia model

Adult male 129/SV mice (18 to 20 g, Taconic farms, Germantown, NY, U.S.A.) were anesthetized with 1.5% halothane for induction and maintained on 1.0% halothane in 70% N₂O and 30% O₂ using a Fluotec 3 vaporizer (Colonial Medical, Amherst, NH, U.S.A.). Ischemia was induced with a 8.0 nylon monofilament coated with silicone resin/hardener mixture (Xantopren and Elastomer Activator, Bayer Dental, Osaka, Japan), as described previously (Hara et al., 1996). The filament was introduced into the left internal carotid artery up to the anterior cerebral artery. For filament withdrawal, the animals were briefly reanesthetized. The MCAO procedures were performed by two investigators (M. E. examined the effects of z-VAD.FMK and MK-801 experiments, whereas M. S. -S. tested z-DEVD.FMK).

Treatment protocol

Both z-VAD.FMK (120 ng) and z-DEVD.FMK (160 ng or 480 ng) were injected intracerebroventricularly (2 μ L; bregma -0.9 mm lateral, -0.1 mm posterior, -3.1 mm deep) either 10 minutes before ischemia or 6, 12, or 18 hours after reperfusion. Control animals were injected with 2 μ L of 0.3% DMSO. Cycloheximide (10 mg/kg) was administered intraperitoneally 1 day before ischemia. MK-801 (3 mg/kg) was administered intraperitoneally 10 minutes before ischemia or 3 or 6 hours after reperfusion. Control animals were injected intraperitoneally with a corresponding volume of PBS. Since hypothermia is a well known consequence of MK-801 treatment, animals were kept in an incubator (ThermoCare System, Incline Village, NV, U.S.A.) at 30 to 31°C for 6 hours after treatment to maintain a body temperature of 37°C. Core temperatures were measured using a thermometer (BAT-12, Physitemp, Clifton, NJ, U.S.A.) after 3 and 6 hours. Control mice (36.8° \pm 0.2°C at 3 hours and 36.7° \pm 0.2°C at 6 hours) did not differ from

MK-801-injected animals ($36.7^{\circ} \pm 0.1^{\circ}\text{C}$ at 3 hours and $36.7^{\circ} \pm 0.2^{\circ}\text{C}$ at 6 hours).

Infarct measurement

For survival longer than 24 hours, infarct size was measured from hematoxylin-eosin (H&E)-stained sections, whereas triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, U.S.A.) stained 2-mm coronal sections were used to assess injury after shorter periods of occlusion. For H&E-stained sections, the brains were first immediately frozen in 2-methylbutane on dry ice and then sectioned. For TTC staining, animals were decapitated, and the brains were divided into five coronal 2-mm sections using a mouse brain matrix (RBM-2000C, Activational Systems, MI, U.S.A.) and stained with 2% 2,3,5-TTC. Infarction volume was quantitated in TTC-stained sections or in H&E-stained cryostat section ($12\ \mu\text{m}$) with an image analysis system (M4, Imaging Research, St. Catharines, Ontario, Canada) and calculated by summing the volumes of each section determined directly (Huang et al., 1994) or indirectly using the following formula: contralateral hemisphere (in cubic millimeters) minus undamaged ipsilateral hemisphere (in cubic millimeters) (Swanson et al., 1990). Differences between "direct" and "indirect" volumes are likely to be accounted for by brain swelling.

Neurologic deficits

Mice were tested for neurologic deficits and scored as described by Bederson and others (1986) with the following minor modifications (Hara et al., 1996): 0, no observable neurologic deficit (normal); 1, failure to extend right forepaw (mild); 2, circling to the contralateral side (moderate); 3, loss of walking or righting reflex (severe). The rater was naive to the treatment protocol and to the group's identity. Assessments were made at 30 minutes after onset of ischemia and 24 and 72 hours (if necessary) after reperfusion.

DNA analysis

Samples were obtained at different time points after 30 minutes of transient MCAO. Ischemic striatal tissue was taken from the third 2-mm section along with homologous tissue from contralateral side after the brain was cut coronally with a brain matrix. For quantitation of DNA damage, a terminal transferase-dependent [^{32}P]ddATP end-labeling method was used (Tilly and Hsueh, 1993) with minor modifications, as described previously (Hara et al., 1997b). Three micrograms of DNA were used in the labeling procedure together with 35 ng of a 100-base pair (bp) DNA fragment as an internal standard. Electrophoresis was performed on the DNA on a 2.0% agarose gel (agarose 3:1, Amresco, Solon, OH, U.S.A.), autoradiographed together with a [^{32}P] standard, and analyzed with the M4 image analysis system. DNA less than 10 kb was used as an index of total DNA fragmentation. To measure oligonucleosomal damage more specifically, ladder DNA less than 1000 bp was measured by summing the areas of each peak (areas under the curve) minus baseline densitometry readings. The method of quantitation was validated using an artificial "smear-ladder" system in which smeared DNA was extracted from a decapitated mouse brain and incubated at 37°C for 48 hours (MacManus et al., 1995b). In a total amount of $1\ \mu\text{g}$ of DNA, 0.02, 0.04, 0.08, and $0.12\ \mu\text{g}$ of a commercially available 200-bp ladder (Invitrogen, Carlsbad, CA, U.S.A.) were added to a constant amount of smeared DNA (Fig. 1). In another experiment, increasing amounts of smeared DNA (0.3, 0.5, 0.7, 0.9 μg) were added to a constant amount of ladder (0.1 μg). The measurements for "total DNA fragmentation" and

"DNA laddering" were related linearly to the amount of total (smeared) and ladder DNA (error < 10%).

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

The TUNEL staining was carried out according to the method of Gavrieli and others (1992) with minor modifications according to Wood and associates (1993). Terminal deoxynucleotidyl transferase and biotinylated dUTP were obtained from Boehringer Mannheim (Mannheim, Germany). The biotinylated dUTP was visualized by the avidin-biotin method with 3'-3' diaminobenzidine as chromogene. The reaction was intensified with 0.04% nickel chloride. For negative controls, either terminal deoxynucleotidyl transferase or biotinylated dUTP was omitted. For positive controls, the sections were treated with DNase I.

For cell counts, sections were counterstained lightly with H&E. Following a systematically random sampling scheme and based on stereologic techniques (Gundersen, 1992; West, 1993; Gómez-Isla et al., 1996), five coronal sections ($12\ \mu\text{m}$) were selected, taken at equally spaced intervals (2 mm) through the full rostrocaudal extent of the brain. The boundaries of the area occupied by TUNEL-positive cells within each section were marked using the Bioquant Image analysis system (Nashville, TN, U.S.A.). The entire volume of the lesion occupied by TUNEL-positive cells was calculated according to the principle of Cavalieri (Cavalieri, 1966). Within each section with TUNEL-positive cells, a systematically random sampling scheme was applied to count cells. The total number of TUNEL-positive cells in each brain was estimated by using ~80 optical dissectors and a $40\times$ objective. Each optical disector was a $100\times 100\ \mu\text{m}$ sampling box with extended exclusion edges. The appropriateness of the sampling scheme chosen was evaluated by calculating the precision of the estimates in each animal, expressed as the coefficient of error (West and Gundersen, 1990). In all cases, the coefficient of error was less than 0.10, suggesting that a minimal amount of variance in the counts can be attributed to the technique. The calculation of total number of TUNEL-positive cells was performed by multiplying the volume density of TUNEL-positive cells by the total volume occupied by TUNEL-positive staining.

Glial fibrillary acid protein immunohistochemistry/TUNEL double staining and CD-45 (leukocyte common antigen) immunohistochemistry

Glial fibrillary acid protein (GFAP)/TUNEL double staining was performed to determine if glial cells show TUNEL staining. The TUNEL staining protocol was performed as described earlier. Subsequently, sections were washed and blocked with 10% normal goat serum in PBS. GFAP double labeling was performed with a rabbit GFAP polyclonal antibody (1:500, Dako Corporation, Carpinteria, CA, U.S.A.) using a three-stage avidin-biotin method and biotinylated goat anti-rabbit IgG as secondary antibody. The reaction product was visualized with 3-amino-9-ethylcarbazole reagent (Elite PK 6101 Kit, Vector Laboratories, Burlingame, CA, U.S.A.). For controls, single GFAP immunostaining was performed in adjacent sections.

To identify inflammatory cells, CD-45 (leukocyte common antigen) immunohistochemical study was performed on adjacent sections with a purified rat anti-mouse antibody (1:500, Clone 30F11.1, Pharmingen, San Diego, CA, U.S.A.) and a three-stage avidin-biotin method with a biotinylated rabbit anti-rat IgG (BA 4001, Vector Laboratories) as secondary antibody.

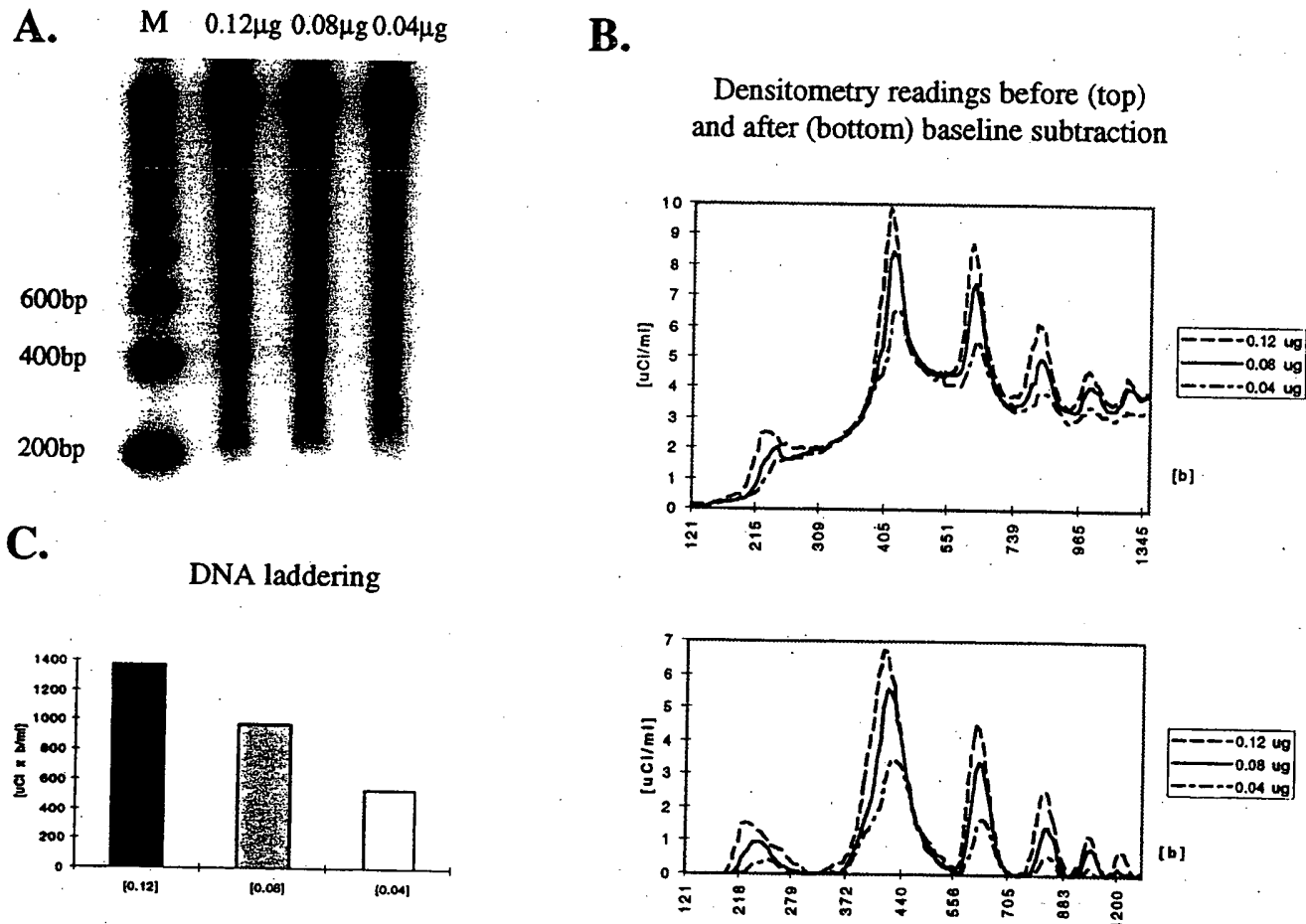


FIG. 1. Quantitative evaluation of "laddered" and "smeared" DNA by agarose gel electrophoresis. To validate the method, decreasing amounts of laddered DNA (0.12 μ g, 0.08 μ g, 0.04 μ g) were added to constant amounts of smeared brain DNA (MacManus et al, 1995b), end-labeled with [32 P]ddATP, separated by agarose gels (A), and compared with a 200-bp standard (M). Each lane was analyzed by densitometry with an image analysis system (B, top). The abscissa represents the length in base pairs [bp]; the ordinate represents μ Ci/mL compared with a radioactive standard (not shown). To demonstrate peak heights corresponding to laddered DNA, a baseline subtraction was performed (B, bottom). The areas under each peak provide a measure of DNA laddering. A linear relation was found between the amount of laddered DNA used in the labeling procedure and the densitometry readings (C). Readings also were linear when increasing amounts of smeared DNA were mixed with a constant amount of laddered DNA (not shown).

Statistical analysis

Data are presented as mean \pm SD. Statistical comparisons were made by one-way analysis of variance and followed by Dunnett test (rCBF) or Tukey test (physiology). For neurologic deficits, Mann-Whitney rank sum test was applied for two groups, and Kruskal-Wallis one-way analysis of variance on ranks followed by Dunn test for three or more groups. For unpaired data (infarction volume and area, DNA laddering, cell counts) two-tailed Student's *t* test was applied. Analysis was made using the software SigmaStat (Jandel Corporation, San Rafael, CA, U.S.A.) or Excel (Microsoft, Redmond, WA, U.S.A.). *P* < 0.05 was considered statistically significant.

RESULTS

Mouse model of mild focal cerebral ischemia

Physiologic parameters before, during, and after ischemia were within the normal range and did not differ between groups. Mean arterial blood pressure remained stable at 110 ± 12 mm Hg, and heart rate at 480 ± 70 beats per minute. Blood gases were stable at pH $7.4 \pm$

0.1, $Paco_2$ at 45 ± 5 mm Hg, and Pao_2 at 125 ± 25 mm Hg. After MCAO, rCBF decreased to 20% of baseline ($20\% \pm 8\%$) and was sustained during 30 minutes of ischemia. The rCBF immediately returned to more than 100% of baseline ($116\% \pm 20\%$) after suture removal.

No evidence for injury was detected 24 or 72 hours (TTC staining) after 10 minutes of MCAO in pilot experiments (*n* = 5 each). Twenty-four hours after 30 minutes of MCAO, ischemic changes were not detected by TTC (*n* = 5) or grossly by H&E (*n* = 5), although infrequent striatal cells showed light microscopic changes consistent with ischemia.

At 72 hours of reperfusion after 30 minutes of MCAO, clear evidence for infarction was found. Infarct size was 32.7 ± 8.3 mm³ (*n* = 17) after vehicle (DMSO) was injected intracerebroventricularly 6 hours after reperfusion. Vehicle injection did not alter infarct size, since noninjected animals showed the same injury volume (31.4 ± 19.2 mm³, *n* = 12). The lesion was located

TABLE 1. Effects of the nonselective caspase inhibitor z-VAD.FMK, the putative caspase 3 inhibitor z-DEVD.FMK, and the NMDA-antagonist MK-801 on infarct volume after 30 minutes of MCAO

Treatment (n)	Rx time (h)	Sacrifice (h)	Infarct volume (mm ³)
Vehicle (9)	6	72	38.1 ± 6.4
z-VAD (8)	6	72	11.2 ± 5.4*
Vehicle (4)	6	7 days	27.1 ± 1.9
z-VAD (4)	6	7 days	9.8 ± 5.0*
Vehicle (6)	18	72	37.5 ± 9.5
z-VAD (4)	18	72	38.3 ± 12.7
Vehicle (7)	before	72	25.0 ± 5.0
z-DEVD (7)	before	72	11.2 ± 2.3*
Vehicle (8)	6	72	26.5 ± 5.3
z-DEVD (9)	6	72	11.2 ± 8.0*
Vehicle (5)	12	72	22.6 ± 5.2
z-DEVD (6)	12	72	15.8 ± 9.2
Vehicle (5)†	before	72	33.4 ± 4.2
MK-801 (5)†	before	72	14.5 ± 5.8*
MK801 (5) †	3	72	29.1 ± 6.2
MK-801 (5) †	6	72	31.1 ± 6.0

Time refers to drug injection after reperfusion except when noted for z-DEVD.FMK and MK-801 (given 10 minutes before MCA occlusion). z-VAD.FMK (120 ng) and z-DEVD.FMK (480 ng) were administered intracerebroventricularly, MK-801 (3 mg/kg) was given intraperitoneally. Data are presented as mean ± SD.

* $P < 0.01$ versus vehicle group.

† MK-801/vehicle-treated mice were held at a constant temperature (31°C) for 6 hours after treatment.

predominantly in the striatum where cells were severely pyknotic. Cortical injury, by comparison, was minor. Lesion volume did not increase significantly by 7 days ($33.5 \pm 21.1 \text{ mm}^3$, $n = 8$, no intracerebroventricular injection), and brain swelling did not contribute significantly to infarct size, as assessed by an indirect method for measurement of infarct size. Cycloheximide (Chex) pretreatment (10 mg/kg, $n = 7$) reduced infarct size ($10.5 \pm 9.0 \text{ mm}^3$ versus $31.4 \pm 19.2 \text{ mm}^3$, $P < 0.05$) at 72 hours, suggesting the importance of protein synthesis inhibition.

Inhibitors of the caspase family reduce infarct size

Table 1 contains the data for experiments using caspase inhibitors and MK-801. Infarct size in vehicle-treated controls were smaller in the z-DEVD.FMK experiments (22.6 to 26.5 mm^3) versus the other two vehicle groups (27.1 to 38.1 mm^3), probably because the

TABLE 2. rCBF during and after 30 minutes of filamentous middle cerebral artery occlusion in z-VAD.FMK and vehicle (0.3% DMSO) groups

	z-VAD.FMK	Vehicle
rCBF (during)	18 ± 4	18 ± 12
rCBF (after)	105 ± 15	105 ± 18

z-VAD.FMK (120 ng) or vehicle were administered intracerebroventricularly in a volume of 2 μL 6 hours after reperfusion following 30 minutes of MCAO. rCBF was measured by Laser-Doppler-flowmetry (expressed as % of baseline). Data represent mean ± SD ($n = 5-6$). There are no significant differences between groups.

TABLE 3. Physiologic variables 10 minutes before and until 1 hour after injections of z-VAD.FMK or vehicle (0.3% DMSO)

	z-VAD.FMK	Vehicle
MABP (before)	115 ± 9	115 ± 6
MABP (after)	112 ± 4	118 ± 12
pH	7.3 ± 0.1	7.3 ± 0.1
Paco ₂	47 ± 5	43 ± 5
Pao ₂	151 ± 17	133 ± 24

z-VAD.FMK (120 ng) or vehicle were administered intracerebroventricularly in a volume of 2 μL 6 hours after reperfusion following 30 minutes of MCAO. Mean arterial blood pressure (MABP, in mm Hg) was measured before and until 1 hour after drug injection. Fifty microliter blood was withdrawn 30 minutes after drug administration. Paco₂ and Pao₂ are given in mm Hg. Data represent mean ± SD ($n = 5-6$). There are no significant differences between groups.

surgical procedure for z-DEVD.FMK was performed by a different investigator (see Methods).

A dose of z-VAD.FMK (120 ng intracerebroventricularly) given 6 hours after reperfusion decreased infarct size (Table 1) and neurologic deficits at 72 hours (1.0 ± 0.9 versus 2.4 ± 0.9 , $P < 0.05$). The protective effects sustained for at least 7 days (Table 1). When given at 18 hours after reperfusion, however, the drug was not effective in reducing gross infarct size (Table 1); neurologic deficits did show some improvements in this group, however (1.2 ± 0.8 versus 2.4 ± 0.9).

A dose of z-DEVD.FMK (480 ng given intracerebro-

TABLE 4. Physiologic parameters before, during and after 30 minutes of ischemia (129/SV mice) after z-DEVD.FMK or vehicle (0.3% DMSO) pretreatment

	z-DEVD.FMK	Vehicle
rCBF (during)	23 ± 7	17 ± 6
rCBF (after)	111 ± 22	101 ± 27
CT (before)	37.0 ± 0.3	36.7 ± 0.9
CT (after)	36.9 ± 0.3	36.4 ± 0.4
CT (72 h)	36.0 ± 1.0	35.2 ± 1.0
MABP (before)	102 ± 11	107 ± 13
MABP (during)	103 ± 12	108 ± 11
MABP (after)	100 ± 14	104 ± 17
HR (before)	494 ± 32	486 ± 36
HR (during)	508 ± 45	461 ± 62
HR (after)	504 ± 25	516 ± 84
pH (before)	7.4 ± 0.1	7.4 ± 0.1
pH (after)	7.4 ± 0.1	7.4 ± 0.1
Pcco ₂ (before)	45 ± 4	45 ± 3
Paco ₂ (after)	48 ± 6	43 ± 5
Pao ₂ (before)	121 ± 12	124 ± 19
Pao ₂ (after)	116 ± 20	130 ± 33

Mean arterial blood pressure (MABP, in mm Hg) and heart rate (HR) were measured before, during and until 30 min after ischemia. Core temperature (CT, in °C) was measured before and until 30 minutes after ischemia and again after 72 hours, just before sacrifice. Fifty microliter blood was withdrawn twice, before ischemia and 30 minutes after reperfusion. Paco₂ and Pao₂ are given in mm Hg. rCBF was measured by Laser-Doppler-flowmetry (expressed as % of baseline). z-DEVD.FMK (480 ng) or vehicle were administered intracerebroventricularly in a volume of 2 μL 10 minutes before ischemia ($n = 5$ each). Data represent mean ± SD. There are no significant differences between groups.

ventriculally 10 minutes before ischemia) significantly reduced infarction volume at 72 hours (Table 1). A lower dose (160 ng) did not reach statistical significance ($18.0 \pm 3.3 \text{ mm}^3$ versus $24.7 \pm 5.0 \text{ mm}^3$, $n = 7$). Infarct size and neurologic deficits (0.4 ± 0.5 versus 1.7 ± 0.5 , $P < 0.05$) were reduced when 480 ng z-DEVD.FMK was administered at 6 hours after reperfusion (Table 1). Infarct volumes, although decreased, did not reach statistical significance when z-DEVD was given 12 hours after reperfusion (Table 1).

The rCBF during ischemia and after reperfusion did not differ between groups (Tables 2 and 4). There were also no significant group differences in physiologic parameters after z-VAD.FMK (Table 3) or z-DEVD.FMK treatment (Table 4).

Treatment with MK-801 significantly reduced infarct size if given before MCAO but not when given 3 or 6 hours after reperfusion (Tables 2 and 3). Neurologic deficits tended to be lower after pretreatment but did not differ from controls when MK-801 was given at 3 or 6 hours after reperfusion (0.4 ± 0.6 [pretreatment], 1.0 ± 0.8 [3 hours], 1.2 ± 0.8 [6 hours] versus 1.0 ± 0.7 [control], $n = 4$ to 5 per group).

DNA fragmentation

The TUNEL-positive cells, present in low numbers throughout the striatum at 24 hours, profoundly increased at 72 hours. The TUNEL-positive cells were identified morphologically as neurons, many of them with nuclear condensation and apoptotic bodies. Almost no TUNEL-positive cells contained GFAP double staining (Fig. 2, $n = 3$ for each time point). At 72 hours, low numbers of CD-45-positive inflammatory cells were found within the vasculature; however, only single cells were located within ischemic brain tissue (not shown).

Laddered DNA, detected first at 24 hours, increased 16-fold at 72 hours and 46-fold at 7 days compared with 24 hours (Fig. 3, $n = 3$ per group). DNA damage was already present at 6 hours as smeared DNA and increased substantially between 24 and 72 hours (Fig 3). Total DNA damage and laddering were significantly inhibited at 72 hours by Chex pretreatment (Figs. 3 and 4).

To test the hypothesis that caspase inhibitors block

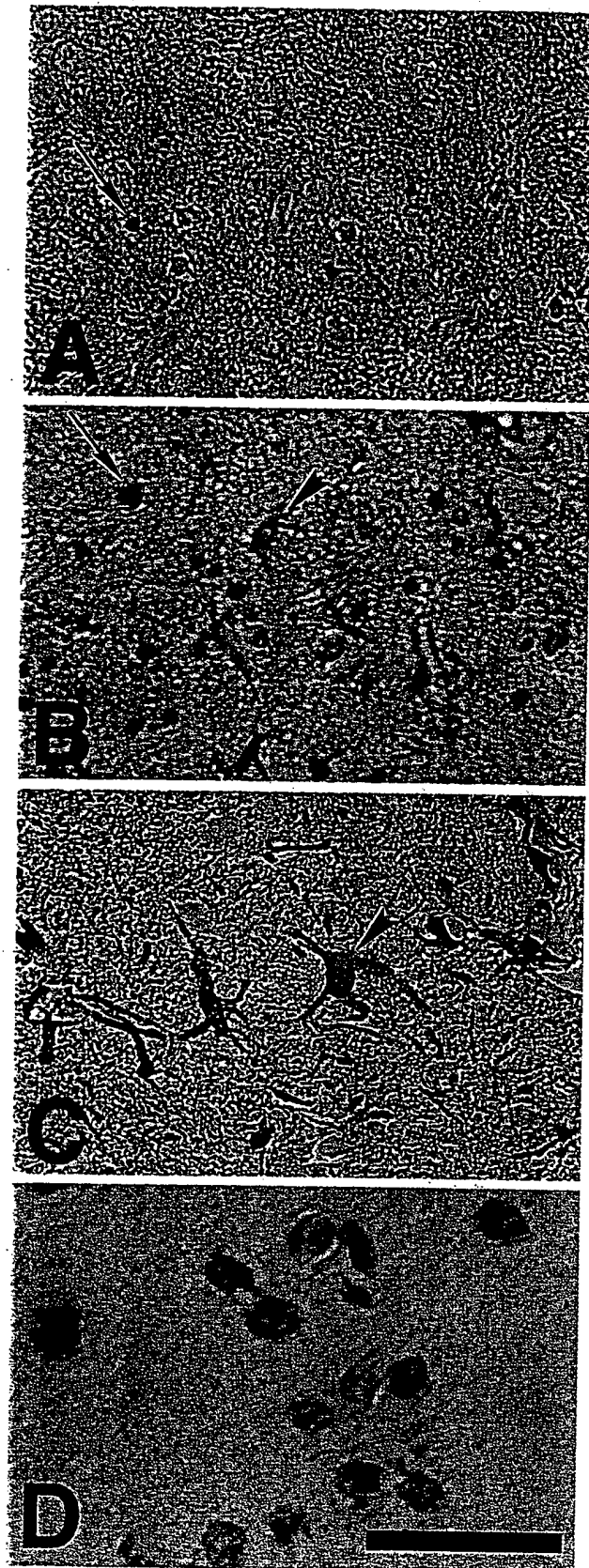


FIG. 2. Time-dependent changes in the appearance of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells and glial fibrillary acid protein (GFAP) staining in striatum after reperfusion following 30 minutes of middle cerebral artery occlusion (MCAO). Tissue sections (6 μm) obtained at 24 hours (A), 72 hours (B), and 7 days (C) were co-stained by TUNEL and GFAP immunohistochemical methods. The TUNEL-positive cells (arrow) were infrequent at 24 hours, increased at 72 hours, and decreased at 7 days. The number of GFAP-positive glial cells (arrowhead) increased over time, but GFAP-labeled cells did not show TUNEL staining (B, C). At a higher magnification (D), TUNEL-positive cells show nuclear condensation and apoptotic bodies (section thickness 40 μm). Scale bar = 50 μm (A, B, C) and 30 μm (D).

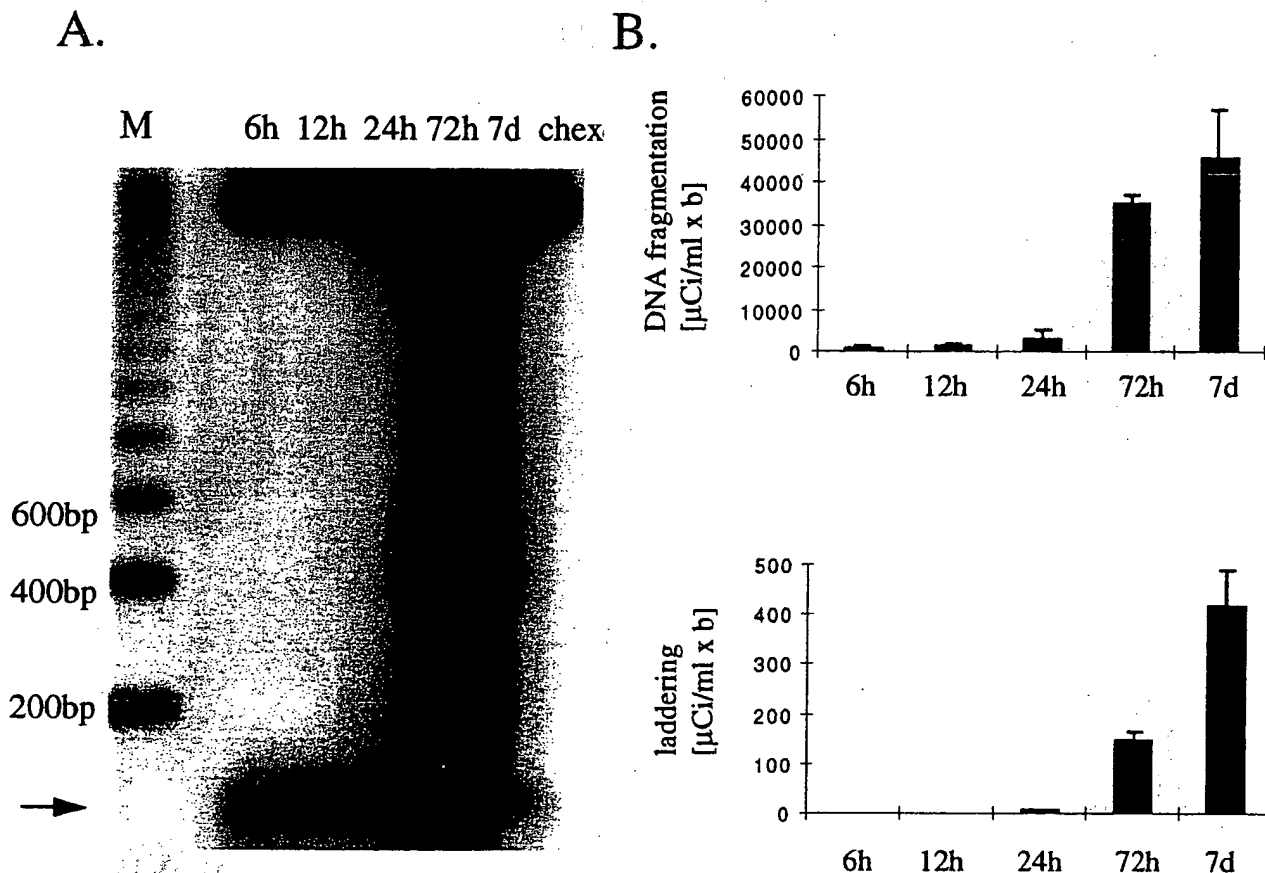


FIG. 3. Cycloheximide (Chex, 10 mg/kg) pretreatment inhibited DNA laddering during reperfusion after 30 minutes of MCAO. DNA was isolated from ischemic striatal tissue after various times after reperfusion, end-labeled with [^{32}P]ddATP, electrophoresed on a 2% agarose gel, and autoradiographed. The autoradiogram (A) shows a 200-bp standard (M) DNA prepared at 6, 12, 24, 72 hours; or 7 days, or at 72 hours after Chex pretreatment. Arrow indicates the location of a 100-bp internal standard. B: Quantitation of "DNA damage" (top) and "DNA laddering" (bottom) was performed as described in Fig. 1. Laddering was first visible after 24 hours and increased by 72 hours (16-fold) and 7 days (46-fold). Each group represents the mean \pm SD of three animals from independent labeling procedures.

apoptosis after ischemia, we compared the number of TUNEL-positive cells in z-VAD.FMK-treated animals (120 ng, given at 6 hours, $n = 4$) versus DMSO ($n = 4$) at 72 hours. Total number of TUNEL-positive cells was significantly reduced in the treatment group ($540,000 \pm 258,000$ versus $1,390,000 \pm 446,000$, $P < 0.05$), and the lesion volume containing TUNEL-positive cells was reduced by 60%. The density of TUNEL-positive cells did not decrease, however ($82,700/\text{mm}^3 \pm 6500/\text{mm}^3$ versus $84,900/\text{mm}^3 \pm 9200/\text{mm}^3$).

A significant reduction in total DNA fragmentation and DNA laddering within ischemic tissue was found at 72 hours in z-VAD.FMK-treated ($n = 4$) and z-DEVD.FMK-treated ($n = 5$) animals (administered 6 hours after reperfusion) compared with controls ($n = 9$) (Figs. 4 and 5), whereas MK-801 pretreatment (3 mg/kg, $n = 4$) did not reduce DNA fragmentation or DNA laddering compared with saline-injected controls ($n = 4$) (Figs. 4 and 5).

DISCUSSION

We provide evidence for the importance of apoptosis to the delayed development of tissue injury after mild

focal cerebral ischemia (30 minutes of MCAO) in the mouse. In this model, ischemic brain damage became grossly visible at 72 hours and did not expand at 7 days after reperfusion. The TUNEL-positive cells and DNA laddering were observed first 24 hours after reperfusion. Cells staining TUNEL-positive probably were neurons, since they did not stain positive for GFAP or CD-45. In fact, Li and colleagues (1995a,b) came to a similar conclusion, since they found GFAP co-staining in only a few TUNEL-positive cells (10%) after 2 hours of MCAO in the rat. Treatment with inhibitors of apoptosis such as cycloheximide, z-VAD.FMK, or z-DEVD.FMK reduced ischemic injury after 30 minutes of MCAO, and peptide methylketones were more effective after mild (30 minutes) than after more prolonged ischemia (2 hours of MCAO). In the 30-minute model, z-VAD.FMK and z-DEVD.FMK decreased infarct volume by 70% and 57%, respectively, whereas the degree of protection was 45% and 35%, respectively, after 2-hour MCAO (Hara et al., 1997a; Moskowitz and Ma, 1997). Peptide methylketones also were more successful many hours (6 hours) after 30 minutes of MCAO compared with 0 to 1 hours after more prolonged (2 hours) MCAO (Hara et al.,

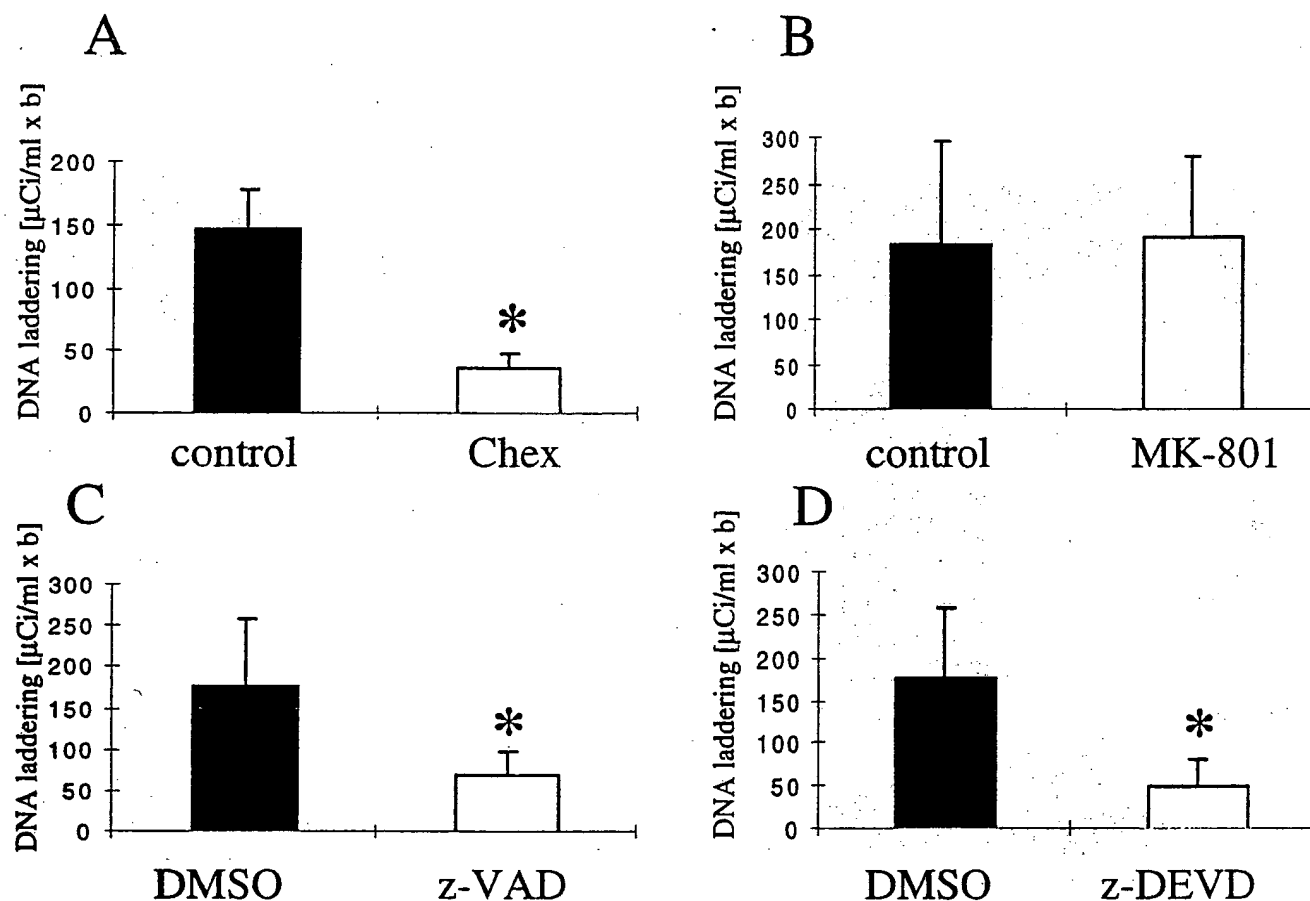


FIG. 4. Cycloheximide (Chex) pretreatment (A), z-VAD.FMK (C), or z-DEVD.FMK (D) given 6 hours after reperfusion, but not MK-801 pretreatment (B) decreased DNA laddering in ischemic tissue. Animals were subjected to 30 minutes of MCAO and reperused for 72 hours. DNA was extracted and separated on agarose gels (see Methods; Figs. 1 and 3). Chex also decreased total DNA damage (3299 ± 696 [drug] versus $35,426 \pm 2099$ [vehicle], $P < 0.01$), as did z-VAD.FMK ($14,394 \pm 8101$ [drug] versus $32,102 \pm 10,353$ [vehicle], $P < 0.05$) and z-DEVD.FMK ($13,341 \pm 5627$ [drug] versus $32,102 \pm 10,353$ [vehicle], $P < 0.01$). Total DNA damage did not differ after MK-801 pretreatment ($24,697 \pm 5229$ [drug] versus $20,264 \pm 8552$ [vehicle]). Units are $\mu\text{Ci}/\text{mL} \times \text{b}$. * $P < 0.05$, each group represents the mean \pm SD of four to nine animals from independent experiments.

1997a; Moskowitz and Ma, 1997). Hence, mild focal cerebral ischemia provides a useful experimental tool to assess the contribution of apoptosis to delayed mechanisms of cell death.

DNA laddering may reflect the consequences of a specific cascade promoting tissue injury after ischemia, which is distinct from necrosis. We found that oligonucleosomal damage was a useful indicator of apoptotic cell death and that a newly developed method for quantitation was reliable and reproducible. A linear relation was found between the amount of ladder DNA used in the labeling procedure and the densitometry readings in validation experiments (Fig. 1). Decreases in ladder DNA did not always accompany decreases in infarct size but depended on whether apoptosis was inhibited. For example, MK-801 pretreatment decreased ischemic damage but not DNA laddering (Figs. 4 and 5). Similar findings were observed after administering 3-aminobenzamide, a widely-used inhibitor of poly(ADP-ribose)polymerase (Endres et al., 1997). On the other

hand, inhibitors of apoptotic cell death such as cycloheximide, z-VAD.FMK, or z-DEVD.FMK clearly inhibited oligonucleosomal DNA damage and decreased infarct size (Figs. 3 through 5; Table 1).

The protective mechanisms of peptide methylketones and MK-801 are different. As observed, MK-801 did not decrease DNA laddering, and its therapeutic window was relatively short in mild ischemia when apoptotic cell death is prominent. We infer that apoptosis probably is not the predominant mechanism of cell death after *N*-methyl-D-aspartate (NMDA) excitotoxicity. Not surprisingly then, z-VAD.FMK and z-DEVD.FMK blocked damage induced by intrastriatal NMDA microinjections only weakly (Hara et al., 1997a). Moreover, TUNEL staining and DNA laddering appear late (24 to 48 hours) after NMDA microinjections (Ayata et al., 1997), suggesting that apoptosis was not primarily a direct NMDA-induced event. Nevertheless, pretreatment with MK-801 protected brain in mild ischemia, suggesting that delayed ischemic death contains both necrotic and apoptotic

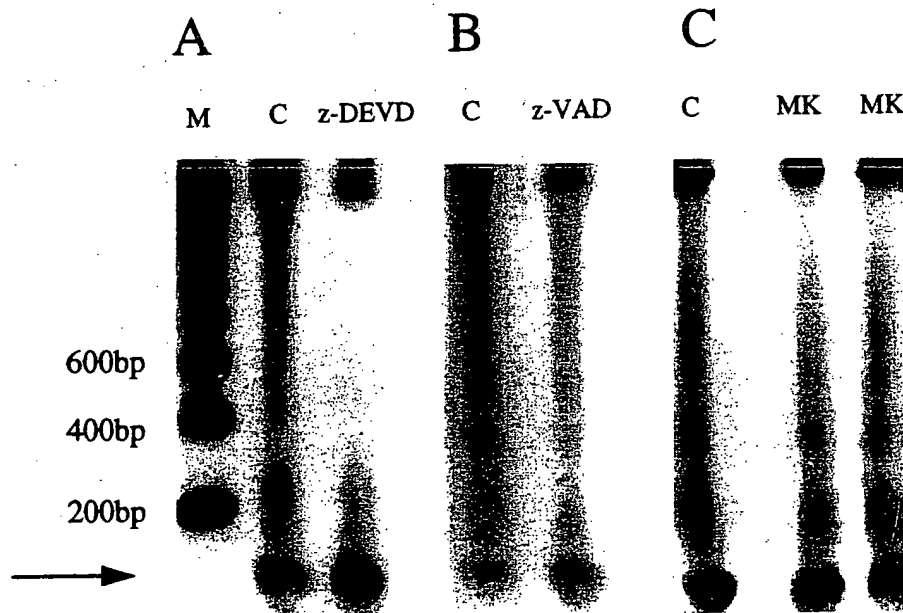


FIG. 5. The z-DEVD.FMK (480 ng) (A) or z-VAD.FMK (120 ng) (B) given intracerebroventricularly 6 hours after reperfusion but not MK-801 pretreatment (3 mg/kg) (C) inhibited DNA laddering within ischemic tissue compared with controls (C). Animals were subjected to 30 minutes of MCAO and 72 hours of reperfusion. DNA laddering was performed as shown (Figs. 1 and 3) and compared with a 200-bp DNA standard (M). The figure shows results for each treatment from representative experiments repeated in four to nine animals. A, B, and C were photographed from separate gels. Arrow indicates a 100-bp internal standard.

mechanisms (as suggested by smeared plus ladder DNA on agarose gels [Figs. 3 and 5]). Because injury is slowly evolving, and MK-801 reduced infarct size when given as a pretreatment, it must mean that NMDA-receptor activation initiates early (within 3 hours) a delayed mechanism of ischemic cell death that is not exclusively apoptosis (DNA laddering does not decrease) and cannot be inhibited by blocking the NMDA receptor after the fact.

The literature supports the notion that MK-801 and caspase inhibitors act through distinct yet complementary and synergistic mechanisms. Choi and colleagues found that NMDA receptor antagonists unmask apoptosis after oxygen-glucose deprivation in neuronal cell cultures *in vitro*. Caspase inhibitors are protective in culture only after MK-801 pretreatment (Gwag et al., 1995). *In vivo*, we demonstrated synergistic protective effects after treatment with MK-801 plus caspase inhibitors (Moskowitz and Ma, 1997). A 35% decrease in infarct size was observed in a mouse model of focal ischemia after combining doses and treatment times when neither compound provided protection alone.

Both the mouse and rat model (30 minutes of ischemia) (Du et al, 1996) show delayed infarct development, prominent DNA laddering, and TUNEL-stained cells. Damage in the mouse, however, was primarily within striatum, whereas injury was mostly within cortex in the rat model. Additionally, the lesion in the mouse did not expand after 3 days as it did in the rat. These differences may relate to greater collateral blood flow in the mouse, as well as differences in vascular anatomy or suture placement, with attendant differences in blood flow reduction. Cycloheximide reduced injury in both species, emphasizing the importance of protein synthe-

sis. The ability of cycloheximide to inhibit apoptosis is well documented in ischemia (Linnik et al., 1993; Du et al, 1996). Cycloheximide may at the same time increase *bcl-2* expression, which also would reduce tissue injury (Furukawa et al., 1997). In general, cycloheximide suppresses synthesis of all proteins, including those that may afford neuroprotection (e.g., superoxide dismutase, growth factors). Hence, it may be difficult to predict the effects of cycloheximide on tissue injury. In our studies, we demonstrate the relevance of caspases to injury development in a model in which cycloheximide reduces infarct size.

Our data could have important implications for the treatment of stroke or other evolving CNS injuries in humans, or even for procedures that carry a risk of brain damage (e.g., neurosurgical procedures or cardiopulmonary bypass). Caspase inhibitors administered at relatively late time points after mild brain injury or ischemia may protect injured tissue. We conclude that inhibitors of ICE family caspases might become valuable drugs to treat stroke in humans.

Acknowledgment: The authors thank Dr. H. Hara for advice.

REFERENCES

- Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J (1996) Human ICE/CED-3 protease nomenclature. *Cell* 87:171
- Ayata C, Ayata G, Hara H, Matthews RT, Beal MF, Ferrante RJ, Endres M, Kim A, Christie R, Waerber C, Huang PL, Hyman BT, Moskowitz MA (1997) Reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knockout mice. *J Neurosci* 17:6908-6917
- Bederson JB, Pitts LH, Tsuji M, Nishimura MC, Davis RL, Bartkowski HM (1986) Rat middle cerebral artery occlusion: evaluation of the

- model and development of a quantification of experimental cerebral infarction in rats. *Stroke* 17:472-476
- Cavalieri B (1966) Geometria degli indivisibili. Torino, Unione Tipografica, Editrice
- Charriaut-Marlangue C, Margail I, Represa A, Popovici T, Plotkine M, Ben-Ari Y (1996) Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis. *J Cereb Blood Flow Metab* 16:186-194
- Dinarello CA (1994) The biological properties of IL-1. *Eur Cytokine Neww* 5:517-531
- Du C, Hu R, Csernansky CA, Hsu CY, Choi D (1996) Very delayed infarction after mild focal cerebral ischemia: a role for apoptosis? *J Cereb Blood Flow Metab* 16:195-201
- Ellis RE, Yuan J, Horvitz HR (1991) Mechanisms and functions of cell death. *Ann Rev Cell Biol* 7:663-698
- Endres M, Wang Z-Q, Namiura S, Waeber C, Moskowitz MA (1997) Ischemic brain injury is mediated by the activation of poly(ADP-ribose)polymerase. *J Cereb Blood Flow Metab* 17:1143-1151
- Fink K, Endres M, Hara H, Waeber C, Moskowitz MA (1997) Early peak IL-1 β formation in transient focal ischemia precedes DNA fragmentation and is inhibitable by caspase inhibitors. *J Cereb Blood Flow Metab* 17(suppl 1):S443
- Friedlander RM, Gagliardini V, Hara H, Fink KB, Li W, MacDonald G, Fishman MC, Greenberg AH, Moskowitz MA, Yuan J (1997) Expression of a dominant negative mutant of ICE in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. *J Exp Med* 185:933-940
- Friedlander RM, Gagliardini V, Rotello RJ, Yuan J (1996) Functional role of interleukin-1 β (IL-1 β) in IL-1 β -converting enzyme-mediated apoptosis. *J Exp Med* 184:717-724
- Furukawa K, Estus S, Fu W, Mark RJ, Mattson MP (1997) Neuroprotective action of cycloheximide involves induction of bcl-2 and antioxidant pathways. *J Cell Biol* 136:1137-1149
- Gavrieli Y, Sherman Y, Ben-sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 199:493-501
- Gómez-Isla T, Price JL, McKeel DW Jr, Morris JC, Growdon JH, Hyman BT (1995) Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *J Neurosci* 16:4491-4500
- Gundersen HJG (1992) Stereology: the fast lane between neuroanatomy and brain function or still only a tightrope? *Acta Neurol Scand* 137:8-13
- Gwag BJ, Lobner D, Koh JY, Wie MB, Choi DW (1995) Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation in vitro. *Neuroscience* 3:615-619
- Hara H, Fink K, Endres M, Friedlander RM, Gagliardini V, Yuan J, Moskowitz MA (1997b) Attenuation of transient focal cerebral ischemic injury in transgenic mice expressing a mutant ICE inhibitory protein. *J Cereb Blood Flow Metab* 17:370-375
- Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang Z, Shimizu-Sasamata M, Yuan J, Moskowitz MA (1997a). Inhibition of interleukin 1 β converting enzyme family proteases reduces ischemic and excitotoxic damage. *Proc Natl Acad Sci USA* 94:2007-2012
- Hara H, Huang PL, Panahian N, Fishman MC, Moskowitz MA (1996) Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. *J Cereb Blood Flow Metab* 16:605-611
- Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA (1994) Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. *Science* 265:1883-1885
- Li Y, Chopp M, Jiang N, Yao F, Zaloga C (1995a) Temporal profile of in situ DNA fragmentation after transient middle cerebral artery occlusion in the rat. *J Cereb Blood Flow Metab* 15:389-397
- Li Y, Chopp M, Jiang N, Zaloga C (1995b) In situ detection of DNA fragmentation after focal ischemia in mice. *Mol Brain Res* 28:164-168
- Li Y, Chopp M, Jiang N, Zhang ZG, Zaloga C (1995c) Induction of DNA fragmentation after 10 to 120 minutes of focal cerebral ischemia in rats. *Stroke* 26:1252-1258
- Linnik MD, Zobrist RH, Hatfield MD (1993) Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. *Stroke* 24:2002-2009
- Liu T, McDonnell PC, Young PR, White RF, Siren AL, Hallenbeck JM, Barone FC, Feuerstein GZ (1993) Interleukin-1 β mRNA expression in ischemic rat cortex. *Stroke* 24:1746-1751
- MacManus JP, Hill IE, Huang ZG, Rasquinha I, Xue Dong, Buchan AM (1995a) DNA damage consistent with apoptosis in transient focal ischaemic neocortex. *Mol Neurosci* 5:493-496
- MacManus JP, Hill IE, Preston E, Rasquinha I, Walker T, Buchan AM (1995b) Differences in DNA fragmentation following transient cerebral or decapitation ischemia in rats. *J Cereb Blood Flow Metab* 15:728-737
- Moskowitz MA, Ma J (1997) Synergistic decrease in infarct volume when an ICE family inhibitor and MK-801 are combined to treat cerebral ischemia. *J Cereb Blood Flow Metab* 17(suppl 1):S434
- Nicholson DW, Ali A, Thornberry NA, Vailancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin TT, Yu VL, Miller DK (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376:37-43
- Relton JK, Rothwell NJ (1992) Interleukin-1 receptor antagonist inhibits ischaemic and excitotoxic neuronal damage in rats. *Brain Res Bull* 29:243-246
- Saito K, Suyama K, Nishida K, Sei Y, Basile AS (1996) Early increases in TNF- β , IL-6 and IL-1 β levels following transient cerebral ischemia in gerbil brain. *Neurosci Lett* 206:149-152
- Swanson RA, Morton MT, Tsao-Wu G, Savalos RA, Davidson C, Sharp FR (1990) A semiautomated method for measuring brain infarct volume. *J Cereb Blood Flow Metab* 10:290-293
- Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM (1995) Yama/CPP32, a mammalian homolog of CED-3, is a crmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose)polymerase. *Cell* 81:801-809
- Tilly JL, Hsueh AJW (1993) Microscale autoradiographic method for the qualitative and quantitative analysis of apoptotic DNA fragmentation. *J Cell Physiol* 154:519-526
- West MJ (1993) New stereological methods for counting neurons. *Neurobiol Aging* 14:275-285
- West MJ, Gundersen HJG (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. *J Comp Neurol* 296:1-22
- Wood KA, Dipasquale B, Youle RJ (1993) In situ labeling of granule cells for apoptosis-associated DNA fragmentation reveals different mechanisms of cell loss in developing cerebellum. *Neuron* 11:621-632
- Yamasaki Y, Matsuura N, Shozuhara H, Onodera H, Itoyama Y, Kogure K (1995) Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. *Stroke* 26:676-681
- Yuan J, Horvitz HR (1990) The Caenorhabditis elegans genes ced-3 and ced-4 act cell autonomously to cause programmed cell death. *Dev Biol* 138:33-41

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)